computational modeling. Time-stamped distances from multiple dye labeling points are statistically resampled and all the possible relative positions of individual domains are mapped geometrically. The choice of a global conformation conforming to all distance constraints is then made given the computed energy profiles. Principal component analysis is applied to such resampled single-molecule trajectories to extract the key conformational dynamics. We apply this resampling scheme to the human insulin-degrading enzyme (IDE), a metallopeptidase potentially implicated in type-2 diabetes. Structurally, IDE possesses two equal-sized N- and C-terminal domains, IDE-N and IDE-C, respectively. IDE is found in the compact closed conformation among all available X-ray structures; yet it processively breaks down insulin to several fragments at non-specific sites. Application of this proposed resampling approach allows one to reconstruct and to discover the unexpected global shearing and grinding motions between IDE-N and IDE-C. This resampling scheme should be applicable for illustrating conformational dynamics of other protein systems.

984-Pos Board B739

A Combined Single Molecule FRET/Magnetic Tweezers Instrument to Calibrate Molecular Tension - Based Fluorescence Probes

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Despite the importance of mechanical forces in biology, few methods are available to measure molecular forces in living systems. To address this issue, we developed molecular tension - based fluorescence microscopy (MTFM), a technique that allows one to visualize piconewton forces using a conventional fluorescence microscope. In MTFM, a flexible linker (polymer) is flanked by a fluorophore and a quencher, such that tension leads to extension of the linker and a 10-30 fold increase in fluorescence. Thus far, MTFM provides the only approach to map piconewton forces at the cell membrane.

To accurately calibrate the conversion from fluorescence to tension, we integrate magnetic tweezers to a total internal reflection fluorescence (TIRF) microscope for single molecule Förster resonance energy transfer (smFRET) - force measurement. To minimize autofluorescence from paramagnetic beads, we use 4.4 micron lambda-DNA fragments to link a MTFM probe to each bead. The setup can apply forces ranging from 1-50 pN to calibrate the probes. Comparing its force-extension data to that of theoretical models, this direct measurement of force-induced extension change provides a good tool for determining cellular force with higher accuracy.

985-Pos Board B740

Farfret: Extending the FRET Range in Single-Molecule Measurements with Multiple Acceptors

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Measurements of Förster resonance energy transfer (FRET) efficiencies at the single-molecule level have become a powerful and invaluable tool as a spectroscopic nanoruler in quantitative studies of biomolecular structures and dynamics for distances between 2 to 10 nm. However, many biological macromolecular complexes and interactions exceed this relatively short range accessible to FRET, thus limiting the great potential of single-molecule FRET methodologies in biological applications. Although recent advances in super-resolution microscopy have paved the way for measuring distances down to 20 nm lateral resolution, there still remains a gap for measuring distances between 10-20 nm.

Here, we present a method termed farFRET that bridges these two scales and extends the conventional range of FRET beyond 10 nm by using the enhanced energy transfer probability of multiple acceptors, the so-called 'antenna effect' (Bojarski et al. J. Phys. Chem. B, 2011, 115, 10120). We combine single-molecule FRET confocal microscopy with pulsed interleaved excitation (PIE) measurements and time-correlated single-photon counting (TCSPC). This allows us to simultaneously measure FRET, stoichiometry and fluorescence lifetime to be able to distinguish farFRET populations with different number of active acceptors.

We show the possibilities of this method on a model system of dsDNA. Our experimental findings are supported by extensive Monte-Carlo simulations. Therefore, a precise distance measurement that surpasses the 10 nm limit is readily available for various future applications.

986-Pos Board B741

Determining Acceptor Dye Quantum Yield from Pulsed Interleaved Single-Pair FRET Measurements

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Single-pair fluorescence resonant energy transfer (spFRET) is a widely accepted technique for determining intra/inter molecular distances on nanometer scale. We present spFRET measurements of freely diffusing DNA constructs using pulsed interleaved excitation (PIE). We engineered a FRET system consisting of two complementary, single stranded DNA oligonucleotides containing either a donor or acceptor fluorophore. The FRET donor was attached to the antisense strand at 12 different positions, each on separate strands. The oligonucleotide containing the FRET acceptor was the sense strand. The two strands were annealed together providing incremental distance changes from 5 to 27 base pairs, by moving the donor fluorophore along the antisense strand every other base pair. We used PIE to sort the species into donor-only, donor-acceptor and acceptor-only species. Average burst intensity from the donor-acceptor population that has been directly excited by the red laser provides a relative measure of the quantum yield of the acceptor dye. Recovery of the quantum yield of the acceptor affords reliable estimates of transfer efficiency, hence inter-dye distances. We find that PIE-mediated sorting produces reliable transfer efficiencies from the donor-acceptor population in the presence of a 20-fold excess of donor-only species.

987-Pos Board B742

Inferring Quantitative Models from Noisy Biophysical Data

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Protein-protein interactions are the basis for all of cellular communication biology. Yet understanding protein complexes is a challenge in living cells because complexes can involve tens of proteins in a region of space limited to a few tens of nanometers in diameter. Currently there is no routine way to determine how many proteins of type X, say, are in a complex. We will demonstrate that it is possible to use superresolution imaging data, from a technique called PALM, to quantitatively determine protein complex stoichiometry. In PALM, proteins of interest are labeled with photoswitchable fluorescing proteins (FPs). In other words, FPs' fluorescence is activated by light. Eventually FPs photobleach (stop fluorescing). Under sufficiently low light, fluorescence signals from different FPs can be separated in time. Each FP can then be individually localized as the center of a point-spread function giving PALM far greater resolution than conventional microscopy. In principle, fluorescence spike numbers over one region of interest (ROI) should coincide with the number of proteins. In practice, FPs blink (fluoresce on and off) before photobleaching. Their blinking properties strongly depend on their local cellular environment. Thus, FP photophysics must be determined while enumerating FPs. This poses a major theoretical problem. Here we propose a statistical inference tool to solve this problem.

988-Pos Board B743

Dual Functioning Genetic Tags for Simultaneous Isolation and Observation of Fluorescent Complexes from Whole Cell Extract

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Single molecule colocalization methods provide facile means of detecting and analyzing biomolecular complex formation. However, many of these complexes can not be reconstituted from purified systems and must be studied in cell extracts. We have developed a new approach for tagging these complexes with small molecule fluorophores (e.g., Cy3 and Cy5) and for isolation of these complexes in situ during a single molecule experiment. We are able to isolate complexes using either immunoaffinity techniques or by biotin/streptavidin interaction. We have used this method to isolate a number of complexes involved in pre-mRNA splicing in yeast including both the U1 and U6 snRNPs comprising 17 and 8 proteins respectively. By introducing multiple tags, we have analyzed protein stoichiometry in these isolated complexes. Finally, we have performed functional assays on the immobilized snRNPs to study their interactions with other components of the RNA splicing machinery in yeast. We predict that this

approach will be broadly applicable for studies of other macromolecular machines.

989-Pos Board B744

Dual Focus Fluorescence Cross-Correlation Spectroscopy for the Investigation of Biomolecule Folding and Binding in Flowing Liquids

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Fluorescence correlation spectroscopy measures the time constants for rate processes giving rise to fluorescence intensity fluctuations observed from one or more microscopic sub-volumes of a nanomolar aqueous solution containing the biomolecules of interest. In our approach, the analyte molecules flow through an electrophoresis capillary under the influence of pressure driven flow or electrophoretic flow and are probed by two spatially offset probe volumes in such a way that the molecules flow sequentially from one probe volume to the next. Fluorescence fluctuations are analyzed from each probe volume independently using autocorrelation analysis, and from the two spatially offset probe volumes using cross-correlation analysis. In this way, we are able to resolve fluorescence fluctuation time constants arising from diffusion, flow, triplet blinking, and conformational fluctuations. Conformational fluctuations are monitored by quenching and unquenching of dye-quencher molecules attached to DNA or RNA hairpin structures and probe the folding and unfolding kinetics of the hairpins. In this presentation, we will discuss recent results that show how base stacking within the loop region of the DNA and RNA hairpins alters the kinetics and thermodynamic stability of the hairpins. We will also discuss binding and unbinding of counterions to individual nucleotides as they flow through the capillary under the influence of an applied electric field. Emphasis will be placed on how the desired information can be extracted using our unique approach to fluorescence correlation spectroscopy.

990-Pos Board B745

A Single-Molecule Study of Toll-Like Receptor 4 Structure and Signalling

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In this study, we use a single-molecule fluorescence approach to image the reorganisation on the surface of live cells of individual fluorescently labelled Toll-like Receptor 4 (TLR4) molecules during signalling. TLR4, a key membrane protein in the innate immune system, is involved in the recognition of microbial pathogens, by detecting the presence of the lipopolysaccharide (LPS) component of exogenous Gram-negative bacteria.
Single-molecule tracking experiments will be described that allow us to follow changes in the diffusion of TLR4 and its oligomerisation state over a period of 30 minutes following addition of LPS. These studies provide new insights into how the TLR4 receptor is organised on the cell surface and cooperatively reorganises on binding LPS to trigger downstream signalling and modulate the immune response.

991-Pos Board B746

Maximizing the Fluorescence Signal and Photostability of Fluorophores by Quenching Dark-States

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Due to its easy detectability fluorescence is widely used in spectroscopy to investigate a variety of chemical and biochemical samples. The characteristics of fluorescence like intensity, lifetime, anisotropy and quantum yield contain information about electronic structure, mobility and orientation of fluorophors. The precision of fluorescence signal is limited by the number of detected fluorescence photons. Furthermore, experiments that require high time resolution for investigations of protein folding and dynamics are generally limited by the photon flux. Hence it is important to investigate and extend the fluorescence photon emission capabilities of fluorophors. In this study, three different additives which enhance the fluorescence signal were investigated as selective quenchers for triplet or radical states of Rhodamine 110 (Rh 110).
Fluorescence correlation spectroscopy (FCS) in combination with power plot analysis was used to describe the entire fluorescence output according to a derived kinetics model for excitation and fluorescence of Rh 110. The application of additives effectively prevented the triplet and radical formation of Rh 110 even at high excitation irradiance in the range of MW/cm^2 leading to more than ten times increased fluorescence countrate. Furthermore we demonstrate that additives also increase the fluorescence signal of labeled biomolecule.

992-Pos Board B747

Scanning Fluorescence Correlation Spectroscopy in Mitochondria of Living Cells

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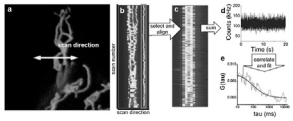
New methods to quantify dynamics and interactions of intracellular species provide key insights in cell biology. Fluorescence Correlation Spectroscopy (FCS) utilizes temporal autocorrelation of fluorescence fluctuations to study the dynamic properties of labeled molecules. Previous studies characterize diffusion and interaction of proteins in the nucleus and cytosol using FCS. However, only few deal with tubular organelles like the endoplasmic reticulum and mitochondria. The ability to accurately place the confocal volume in these dynamic organelles limits point FCS in vivo. Originally applied in membranes, scanning FCS (SFCS) addresses these challenges.
We applied SFCS to measure concentration and dynamic properties fluorescently-labeled molecules in mitochondria. SFCS accurately positions the confocal volume (in the x-y plane) by moving it along a linear scanning path. At a scanning orientation perpendicular to the mitochondria, we can also reduce photobleaching due to the brief residence times in the confocal volume.



General scheme of SFCS. (a) shows a fluorescence image of a mitochondria. The scan direction is oriented perpendicular to the mitochondria. (b) shows the acquired data before selection of mitochondria(orange box) and alignment(c). (d) represents the fluctuation trace, which is correlated and fitted to diffusion models (e).

993-Pos Board B748

Parallel Single-Molecule Excitation Spectroscopy of Gold Nanorods

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Gold nanorods show intense two photon luminescence arising from surface plasmon resonances (SPRs). Such SPRs exhibit a strong dependence on the environmental conditions and can be used to detect interactions between a nanorod and single biomolecules. Here we report a novel technique to acquire excitation spectra of multiple single gold nanorods in parallel, within a few seconds. We acquired two-photon excitation spectra of tens of single gold nanorods, and analyzed individual gold nanorod features. 3-dimensional fitting yields a spectral resolution of 1 nm. This allows for discriminating between single and multiple gold nanorods, as well as analysis of spectral changes in time. Using this technique we aim to detect single protein-gold nanorod interactions within a living cell.

994-Pos Board B749

Single-Cell Single-Molecule Co-IP Analysis

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Recent advances in single molecule imaging technique, such as single molecule Co-IP, have allowed us to probe interactions between weakly binding